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STUDIES OF HUMAN PLACENTAL AROMATASE

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SUMMARY

Kinetic analysis of aromatization of androstenedione (Δ^4) indicates that 3 mol of oxygen and NADPH are consumed in the formation of estrone (E1). During the steady state, the 3d oxidation appears to be the rate limiting step since both 19-hydroxy and 19-oxo-androstenedione accumulate prior to E1. Cytochrome P-450 participation in each hydroxylation step is suggested by (1) inhibition by known P-450 inhibitors other than CO, (2) inhibition by an antibody to NADPH-cytochrome *c* reductase, (3) spectral studies of P-450 binding of substrate and intermediates, and (4) solubilization and partial resolution of enzyme components using digitonin and DEAE cellulose. A survey of steroidal compounds has revealed that 5α-reduced androstenedione is a potent competitive inhibitor which may be of physiologic importance in control of ovarian estrogen synthesis. Finally, many steroidal drugs used in treatment of breast cancer are competitive inhibitors. The non androgenic compound 1-ene-testololactone is effective both *in vitro* and *in vivo* suggesting that antitumor activity of steroidal drugs may be due to inhibition of estrogen synthesis rather than direct androgen action.

INTRODUCTION

On the basis of indirect evidence it has been inferred that the aromatization of androstenedione (Δ^4)* proceeds by a reaction sequence similar to that depicted in Fig. 1. Although it has been generally assumed that three or more hydroxylation reactions are involved, our lack of knowledge concerning the stoichiometry of the reaction has made it difficult to rule out alternative pathways involving other types of reactions or other sequences. In addition, little was known concerning the relative rates of the intermediate reactions or the nature or number of the enzymes involved.

For these reasons we have carried out a number of experiments designed to determine the stoichiometry of the reaction, elucidate the nature of the enzymes involved, as well as contribute to our rather superficial knowledge of the mechanism of aromatization. In addition, we have tested a number of compounds as aromatase inhibitors in an effort to gain insight into mechanisms whereby estrogen production may be controlled under both physiological as well as pharmacological conditions.

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* Abbreviations used in the text are as follows: androstenedione, Δ^4; luteinizing hormone, LH; pregnant mare serum gonadotropin, PMSG; human chorionic gonadotropin, HCG.

EXPERIMENTAL

Microsomes were prepared from freshly delivered human term placentae and stored frozen in 0·05 M potassium phosphate buffer pH 7·4. Oxygen consumption was measured using a Clark-Yellow Springs Instruments O_2 monitor which permitted periodic withdrawal of aliquots for enzyme assay. Spectrophotometric experiments were performed using an Aminco Chance Dual Wavelength-Split Beam Spectrophotometer. The original procedure of Omura and Sato [1] for the determination of cytochrome P-450 was modified in order to minimize spectral perturbations caused by contaminating hemoglobin [2]. Three ml of a freshly prepared microsomal suspension which had been gently gassed with CO were added to both sample and reference cuvettes and a baseline was obtained. A few crystals of $Na_2S_2O_4$ were then added to the sample cuvette resulting in the formation of reduced cytochrome P-450-CO complex. Cytochrome P-450 content was then determined from the difference in displacement at 450 and 490 mm using a millimolar extinction coefficient of 100 [2]. Aromatase activity was estimated using the tritiated water technique which is based upon the loss to H_2O of the 1β and 2β protons of androstenedione or testosterone when these steroids labeled with tritium in

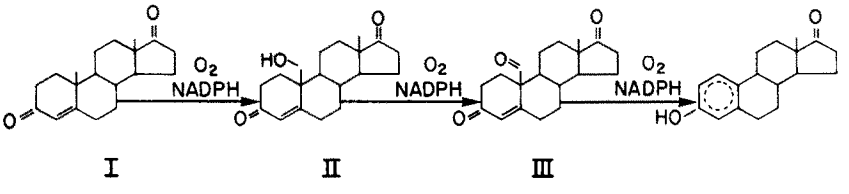

Fig. 1. Proposed mechanism of aromatization.

the 1 and 2 positions are converted to estrogens [3]. The development of this rapid new assay technique has made it possible to study in more detail the kinetic parameters of the aromatase complex including studies related to the stoichiometry of the overall system.

RESULTS

Stoichiometry of aromatization

The addition of androstenedione to microsomes containing NADPH resulted in a marked increase in the rate of O_2 consumption which was constant for only a few minutes after which the rate slowly decreased. In contrast, the rate of aromatization of androstenedione remained constant for at least 30 min. Increased oxygen utilization required the presence of NADPH and appeared to be specific for aromatase substrates. The addition of reaction products–estrone or formate, ethanol, or dissimilar steroids such as progesterone had no effect. Comparison of the initial rate of O_2 consumption with the rate of aromatization (Table 1) revealed that 5–12 mol of O_2 are consumed per mol of estrogen formed during the early stages of the reaction, the ratio varying from one microsomal preparation to another. These high and variable ratios suggested that some oxygen-requiring reaction(s) other than aromatization is also stimulated by the addition of androstenedione to placental microsomes.

Attempts were made to selectively inhibit O_2 consumption without inhibiting aromatization. Of several known oxygenase inhibitors tested, only KCN proved to be effective for this purpose. As can be seen in Table 1, addition of 4 mM KCN to the reaction mixture greatly inhibited O_2 consumption without significantly reducing the rate of aromatization. Under these conditions the stoichiometry of O_2 consumption to estrogen formation approached 3, that is, 3·2 mol of O_2 reduced per mol of estrogen formed. Higher concentrations of KCN inhibit O_2 consumption as well as aromatization (data not shown), but under these conditions the stoichiometry remained at 3. Thus demonstrating that three O_2 requiring reactions are indeed required for the aromatization of androstenedione.

In order to determine if the three oxygen requiring reactions of aromatization are catalyzed by a mixed function oxidase(s), NADPH consumption by placental microsomes in the presence of androstenedione was also investigated. As shown in Table 1, for a given microsomal preparation, the rate of NADPH oxidation was equal to the rate of O_2 consumption. Furthermore, in the presence of KCN, the rate of androstenedione-stimulated NADPH oxidation was inhibited to the same extent as was O_2 consumption. Under these conditions, the net NADPH oxidation was 3 times the estrogen production, thus indicating that 3 mol of NADPH as well as 3 mol of O_2 were required for the production of 1 mol of estrogen, consistent with the reaction sequence proposed in Fig. 1.

The nature of the KCN-sensitive O_2 consuming reaction

As shown in Table 1, in the absence of KCN, 9 mol of O_2 were required for the formation of one mol of estrogen. Since 19-hydroxy-androstenedione (19-OH-Δ^4) and 19-oxo-androstenedione (19-oxo-Δ^4) were known intermediates in aromatization, experiments were designed to determine if accumulation of these hydroxylated intermediates during the early stages of the aromatization reaction might account for this extra O_2 consumption.

In an initial experiment androstenedione was incubated with placental microsomes in the presence of NADPH. As shown in Fig. 2, a lag in estrogen production occurred during the first minute of the reaction as previously reported by Wilcox and Engel [4]. The hydroxylated intermediates, 19-hydroxy-androstenedione and 19-oxo-androstenedione, were also isolated and their rates of formation during the first minutes of the reaction were determined. As shown in Fig. 2, the rate of synthesis of 19-hydroxy-androstenedione was greater than that of 19-oxo-androstenedione which in turn was greater than the rate of estrogen production. The appearance of 19-oxo-androstenedione subsequent to formation of 19-hydroxy-androstenedione and prior to estrogen formation (at 0·5 min.) is consistent with the suggestion that the 19-oxosteroid, or its hydrated equivalent the 19-gem diol, is the second intermediate in aromatization.

In order to determine to what extent the accumulation of these intermediates might account for the 5–10 mol O_2 normally consumed in the production of 1 mol estrogen, the following experiment was performed. Placental microsomes (12 mg protein/ml)

Table 1. Utilization of NADPH and O_2 by placental microsomes in the presence of androstenedione

Additions	Nanomoles/min/mg protein			Stoichiometry	
	O_2	NADPH	Estrogen	O_2 Estrogen	NADPH Estrogen
None	0·313	0·336	0·035	9·0	9·6
4 mM KCN	0·086	0·087	0·027	3·2	3·2

NADPH oxidation was measured in 1·0 ml containing 31 nmoles NADPH, 106 nmoles androstenedione, and 1·4 mg protein. O_2 consumption was measured in 2·5 mls containing 265 nmoles androstenedione, 17·5 mg protein, and an NADPH regenerating system.

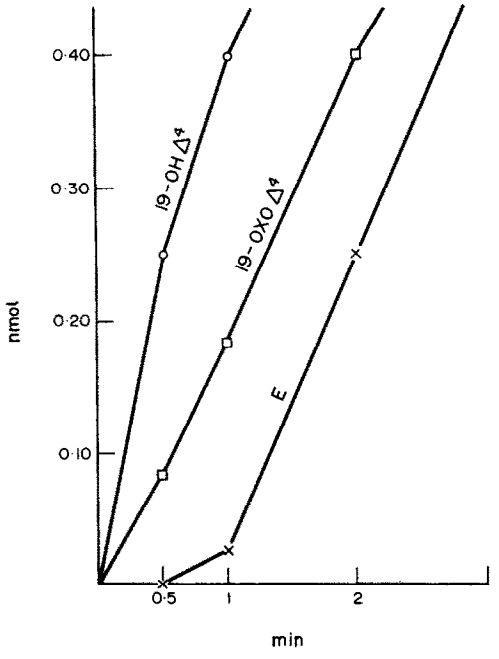

Fig. 2. Rates of formation of products and intermediates of the aromatase reaction at early time. The reaction mixture contained 2·0 mg protein, 35 nm androstenedione (Δ^4), and a NADPH regenerating system in 3·0 ml buffer.

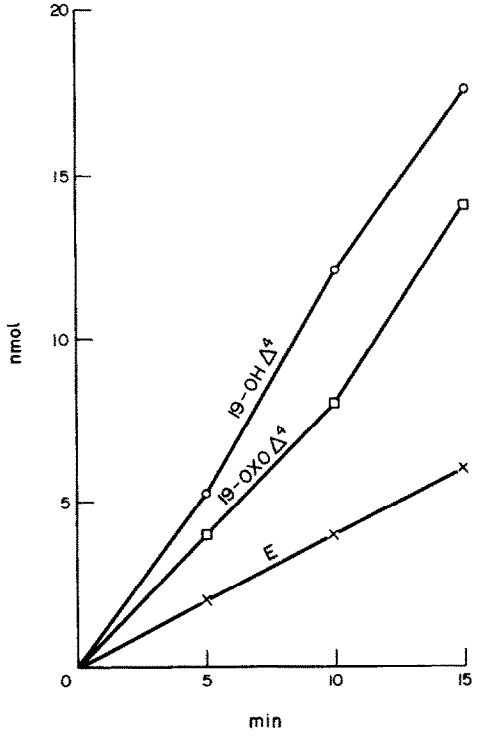

Fig. 3. The rates of formation of products and intermediates of the aromatase reaction during steady state. The reaction mixture contained 36 mg protein, 855 nm androstenedione (Δ^4), and a NADPH regenerating system in 3·0 ml.

were incubated with 285 μM androstenedione in the reaction chamber of the O_2 monitoring device. O_2 consumption was monitored after the addition of an NADPH regenerating system and aliquots were periodically removed and products and intermediates were chromatographically resolved and quantitated. By assuming that the linear sequence depicted in Fig. 1 obtains, a minimal estimate of the rate of formation of any intermediate can be obtained by summing the rate of formation of that intermediate plus the rate(s) of formation of all products derived from that intermediate. Using this approach it was determined, as shown in Fig. 3, that the rate of formation of 19-hydroxy-androstenedione was greater than that of 19-oxo-androstenedione which was in turn greater than the rate of estrogen formation. Furthermore, the amount of O_2 required to produce each of the intermediates and products was calculated assuming 3 mol O_2/mol estrogen, 2 mol O_2/mol 19-oxo-androstenedione, and 1 mol O_2/mol 19-hydroxy-androstenedione. When this theoretical O_2 consumption is compared to the amount of O_2 consumed in 10 min. (Fig. 4) it can be seen that the calculated O_2 consumption was essentially equal to the O_2 consumption as experimentally determined.

The addition of 10 mM KCN to the reaction mixture greatly altered the distribution of intermediates observed in the absence of KCN. While the rate of estrogen production was decreased only slightly ($\simeq$ 25%), 19-oxo-androstenedione formation was reduced to the extent that it could not be detected while 19-hydroxy-androstenedione was present only in trace amounts. In this case, very little of the O_2 consumed resulted in the formation of free intermediates as shown in Fig. 4, and only 3·2 mol O_2 were consumed per mol of estrogen formed.

These experiments confirmed our initial observation that 3 mol of O_2 and 3 mol of NADPH are required for the production of one mol of estrogen and demonstrated that the rapid initial rate of O_2 consumption was due to the formation of intermediates. Our data further indicate that in the presence of excess androstenedione and absence of KCN the rate of formation of 19-hydroxy-androstenedione was

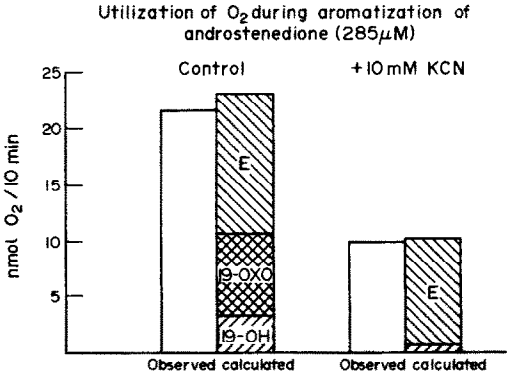

Fig. 4. The reaction was carried out in the O_2 analyzer using the conditions described in Fig. 3. After 10 min, the reaction was terminated and all products and intermediates were chromatographically resolved and quantitated. Theoretical O_2 consumption was calculated as described in the text.

greater than the rate of formation of 19-oxo-androstenedione which in turn was greater than the rate of estrogen production. Therefore, under these conditions, the final step in aromatization (the conversion of 19-oxo-androstenedione to estrone) appears to be rate limiting. In the presence of KCN, however, the rates of all three hydroxylation reactions are approximately equal.

The role of cytochrome P-450 in aromatization

This was suggested by the observed stoichiometry of 1 mol NADPH oxidized per mol O_2 reduced. The lack of inhibition by CO or metyrapone [5, 6] seemed to suggest that this hemoprotein was not involved; however, two other known cytochrome P-450 inhibitors, aminoglutethimide and SKF 525-A, were potent aromatase inhibitors [6]. Furthermore, Estabrook *et al.* had demonstrated that a decreased rate of electron flux from NADPH could result in loss of CO inhibition in systems which were normally CO sensitive [7]. For these reasons, we felt that lack of CO inhibition of aromatization of androstenedione was not necessarily conclusive proof of lack of cytochrome P-450 involvement. Therefore, immunochemical and spectrophotometric studies were carried out in an attempt to resolve this enigma. These studies, described elsewhere in detail [6, 8], are summarized below.

Using an antibody prepared by Dr. B. S. S. Masters, which specifically inhibits cytochrome P-450 reductase reactions, we have observed potent inhibition of aromatization of androstenedione, 19-hydroxy-androstenedione, 19-oxo-androstenedione, and 19-nortestosterone. The nature of this inhibition is such that the initial reduction of cytochrome P-450 appears to be rate limiting during the aromatization of androstenedione, but not rate limiting during the aromatization of 19-nortestosterone. This observation explains the lack of CO inhibition of the former reaction, since as mentioned above, CO inhibition does not obtain when the supply of electrons from NADPH is limiting [7].

Additional evidence of cytochrome P-450 involvement was obtained from binding studies using placental microsomal cytochrome P-450 [6, 8]. As briefly summarized in Table 2, we determined that steroids which are substrates or intermediates for estrogen biosynthesis or inhibitors of aromatization bound to cytochrome P-450. This absolute binding specificity confirms the role of microsomal cytochrome P-450 in estrogen biosynthesis. Furthermore, high affinity binding of 5α-reduced steroids to cytochrome P-450 and consequent inhibition of aromatization suggested a possible role for these steroids in the control of estrogen biosynthesis.

Aromatase inhibitors

In order to explore this possibility, a series of experiments in which steroidal substrate analogs were tested for their ability to inhibit the aromatase were carried out. Using the ^3H_2O assay and 1 μM androstenedione, various steroids were tested for their ability to inhibit at 10 or 100 fold higher concentrations than substrate. In general, the results obtained [9] were similar to those recently published by Schwarzel *et al.*[10]. The most interesting finding, as discussed above, was the marked inhibitory activity of 5α-reduced C_{19} steroids as compared with their 5β reduced counterparts (Table 2). This difference together with the potency of 5α-androstanedione, which binds to the enzyme with nearly the same affinity as does testosterone, raised the possibility of a physiologic control mechanism for estrogen synthesis. For example, estrogen secretion by the ovary of many mammalian species including the human falls precipitously shortly before ovulation. Since androstenedione is a major substrate for both 5α-reductase and aromatase, the amount of estrogen formed could depend upon the relative activity of both of these enzymes as seen in Figure 5. For example, induction of 5α-reductase, perhaps by LH, could serve to reduce the rate of aromatization (1) by reducing available androstenedione and (2) by increasing the intracellular concentration of 5α-reduced aromatase inhibitors. This attractive mechanism could account for the previously unexplained abrupt decline in estrogen synthesis prior to ovulation, or could contribute to follicular atresia by removing the follicle stimulating effect of estrogen.

Table 2. Cytochrome P-450 binding and inhibition of aromatization of androstenedione by various naturally occurring steroids

Steroid*	Cytochrome P-450 binding	% Inhibition
Testosterone	Type I	88
19-Nortestosterone	Type I	80
5α-Androstanedione	Type I	88
5α-Dihydrotestosterone	Type I	82
Androsterone	Type I	40
5β-Androstanedione	None	0
5β-Dihydrotestosterone	None	2
Estrone	None	0
Progesterone	None	0
Dehydroepiandrosterone	None	10

* For inhibition studies a concentration of inhibitor was utilized which was 10 fold greater than the concentration of androstenedione.

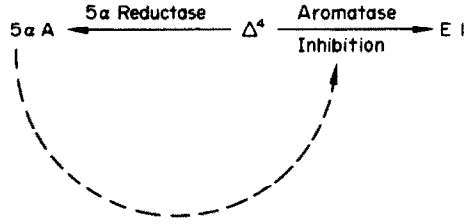

Fig. 5. Proposed mechanism whereby 5α-reductase might serve to control aromatase activity.

We have carried out preliminary experiments to determine the conversion of tritiated androstenedione to estrogens and 5α-reduced steroids in immature rat ovaries following PMSG and HCG stimulation. The results shown in Table 3 indicate that 24 h following injection of PMSG (100 I.U., S.C.) a 50% decrease in 5α-reductase activity had occurred while aromatase activity was greatly increased. However, 6 hr after injection of HCG (100 I.U., S.C.) 5α-reductase activity returned to levels greater than those observed in untreated animals, while aromatase activity was reduced 80%. This situation was greatly altered by 24 h after HCG stimulation. As shown in Table 3, 5α-reductase activity had decreased to 36% of control while aromatase activity had risen to its highest level. Although not definitive, these results strongly suggest that a reciprocal relationship between 5α-reductase and aromatase activity may be of physiologic significance in the control of ovarian estrogen synthesis.

The possible significance of inhibition of estrogen synthesis as a mechanism underlying androgen therapy in human breast cancer also was of interest. The concept of contra-hormone treatment of hormone dependent tumors was utilized by Huggins who showed that estrogen treatment can produce remissions of prostatic tumors in man [11]. This concept was applied to breast cancer in 1939 by Ulrich [12] who produced remissions of breast cancer in two women by treatment with testosterone. Following additional reports indicating beneficial effects of androgen therapy in breast cancer, a massive effort was put forth to obtain active testosterone derivatives which were lower in or devoid of virilizing activity. Of the many compounds tested, 5α-dihydrotestosterone and various substituted derivatives proved to be effective. Of particular interest was the development of the compound 1-ene-testololactone (Teslac) which retains breast tumor activity, but is essentially devoid of androgenic activity [13]. Clearly, the antibreast tumor activity of this drug cannot depend on a direct androgenic effect on the tumor cells. While several alternative possibilites may explain its mode of action, we have considered the possibility that Teslac, and other active androgen derivatives may act by inhibiting estrogen synthesis. It can be seen in Table 4 that in addition to Teslac a number of other steroidal drugs commonly used in the treatment of breast cancer were aromatase inhibitors *in vitro* although none was as active as 5α-androstanedione. Furthermore, when Teslac was administered to two male subjects with gynecomastia 50–80% inhibition of the peripheral conversion of circulating androstenedione to estrone was observed (Table 5). Reduction of peripheral estrogen synthesis during treatment led to marked clinical improvement in each subject [14].

Table 3. Aromatase and 5α-reductase activity in ovaries of gonadotropin stimulated immature rats

Treatment	Nanomoles/h Aromatase	Nanomoles/h 5α-Reductase
None	0·00	0·313
24 h PMSG	0·020	0·170
24 h PMSG followed by 6 h HCG	0·004	0·470
24 h PMSG followed by 24 h HCG	0·118	0·095

All rats were sacrificed at 28 days of age. Ovaries were removed, minced, and incubated in Medium 199 containing 1 μM [1,2-^{3}H]-androstenedione. Reactions were carried out for 1 h at 37°C under an atmosphere of 95% O_2, 5% CO_2.

Table 4. Inhibition of aromatization by various steroids and steroidal drugs

Compound	% of control
Dihydrotestosterone	8
17α-Methyltestosterone	32
2α,17α-Dimethyltestosterone	52
7α,17α-Dimethyltestosterone	18
2-Hydroxymethylenedihydrotestosterone	43
7α-Methyl-19-Nortestosterone Acetate	23
2α-Hydroxytestosterone Dipropionate	81
1-ene-Testololactone (Teslac)	48
6-Dibromomethylenetestosterone Propionate	51
Testosterone Propionate	41

Table 5. *In vivo* inhibition of aromatization by Teslac

Patient	Treatment	% Aromatization androstenedione
JK (10 yr)	None	55
	Dexamethasone	58
	Teslac	20
JS (43 yr)	None	4·7
	Teslac	0·4

DISCUSSION

Our experiments have revealed that in the presence of NADPH and saturating concentrations of androstenedione, placental microsomes consumed large quantities of O_2, only a portion of which could be accounted for on the basis of estrogen formed. The balance of this O_2 appeared to be utilized in the formation of hydroxylated intermediates which were not converted to estrogen during the course of the reaction. In the presence of KCN, however, such intermediates did not accumulate with the result that all of the O_2 consumed was utilized for the production of estrogens. Under these conditions, 3 mol of O_2 were consumed per mol of estrogen formed.

The initial rate of NADPH oxidation was equal to the initial rate of O_2 consumption both in the presence and absence of KCN. This indicates that 1 mol of NADPH was oxidized per mol O_2 consumed and 3 mol of NADPH were oxidized per mol of estrogen formed. Therefore, the stoichiometry of the overall reaction is established as $3\,O_2$:3 NADPH:1 estrogen. This stoichiometry obtains both in the presence and absence of KCN and substantially confirms the sequence proposed in Fig. 1. Our data indicate that no significant conversion of androstenedione to estrone can occur by any mechanism requiring less than three hydroxylation reactions.

We have further observed that in the presence of excess androstenedione, the rate of formation of 19-hydroxy-androstenedione is greater than that of 19-oxo-androstenedione which is greater than that of estrone. This suggests that the rate determining step in the aromatization of androstenedione is the third hydroxylation. In the presence of KCN, however, the first two hydroxylations appear to be inhibited to a greater extent than the third. This inhibition of non-rate determing steps in the reaction resulted in greatly decreased O_2 consumption concomitant with only slight inhibition of aromatization. In the presence of 10^{-2} M KCN, free intermediates no longer accumulated and all of the O_2 and NADPH consumed could be accounted for on the basis of estrogen formed.

The observed stoichiometry of 1 mol NADPH oxidized per mol O_2 reduced suggested the involvement of cytochrome P-450 in aromatization. This was confirmed by inhibition of aromatization by an antibody specific for cytochrome P-450 mediated reactions. We have further observed that placental microsomal cytochrome P-450 has absolute specificity for aromatase substrates, intermediates, and inhibitors. These studies also point to the existence of a new class of cytochrome P-450 mediated reactions whose kinetic mechanism differs somewhat from that observed in liver. In such reactions CO inhibition may not be detectable thus demonstrating that greater care must be exercised in the assignment of cytochrome P-450 involvement based solely upon CO inhibition studies.

Considering the high degree of specificity of placental microsomal cytochrome P-450, it seemed possible that high affinity binding of 5α-androstanedione and dihydrotestosterone (steroids that are not aromatized) may have some physiological significance. Initial experiments using the PMSG stimulated immature rat ovary have revealed that indeed a reciprocal relationship appears to exist between 5α-reductase and aromatase activities. At present, it is not clear whether decreased aromatization is due to depletion of androstenedione or production of inhibitory 5α-reduced metabolites of androstenedione. Nevertheless, induction of 5α-reductase by LH appears to be an attractive mechanism to explain the precipitous drop in estrogen production which occurs during the mid-cycle LH surge in humans.

In addition to 5α-reduced steroids which inhibit aromatization in physiological concentrations, we have also investigated a number of steroidal drugs. One of the most interesting of these compounds is Teslac, which is capable of inducing breast cancer regression even though the steroid lacks androgenic potency. Teslac, and other antibreast cancer drugs are capable of inhibiting estrogen biosynthesis *in vitro*. The striking inhibition of peripheral aromatization by Teslac has led us to consider the possibility that the antitumor activity of all these steroidal drugs may be due not to their androgenic effect, but rather to their ability to decrease peripheral conversion of androstenedione to estrone.

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REFERENCES

1. Omura T. and Sato R.: *J. biol. Chem.* **239** (1964) 2370–2378.
2. Estabrook R. W., Peterson J. A., Baron J. and Hildebrandt A. G.: In *Methods in Pharmacology* (Edited by C. F. Chignell). Academic Press, New York Vol. 2 (1972) pp. 302–350.
3. Thompson E. Aubrey Jr. and Siiteri Pentti K.: *J. biol. Chem.* **249** (1974) 5364–5372.
4. Wilcox B. R. and Engel L. L.: *Steroids Supplement* I, (1965) 49–57.
5. Meigs R. A. and Ryan K. J.: *J. biol. Chem.* **246** (1971) 83–87.
6. Thompson E. Aubrey Jr and Siiteri Pentti K.: *Ann. New York Acad. Sci.*, **212** (1973) 378–391.
7. Estabrook R. W., Franklin M. R. and Hildebrandt A. G.: *Ann. New York Acad. Sci.* **174** (1970) 218–232.
8. Thompson E. Aubrey Jr. and Siiteri Pentii K.: *J. biol. Chem.* **249** (1974) 5373–5378.
9. Thompson E.: Doctoral Dissertation, Univ. Texas South-Western Medical School, Dallas, Texas (1973).
10. Schwarzel W. C., Kruggel W. G. and Brodie H. J.: *Endocrinology* **92** (1973) 866–881.
11. Huggins C. and Hodges C. V.: *Cancer Res.* **1** (1941) 293–304.
12. Ulrich P.: *Acta Un. Int'l. Cancer* 4 (1939) 377–384.
13. Segaloff A.: *Recent Prog. Horm. Res.* **22** (1966) pp. 351–374.
14. Hemsell D., Siiteri P. K. and MacDonald P. C.: In press.